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Genetic and biochemical characterization of ISP6, a small mitochondrial outer membrane protein associated with the protein translocation complex

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To search genetically for additional components of the protein translocation apparatus of mitochondria, we have used low fidelity PCR mutagenesis to generate temperature-sensitive mutants in the outer membrane translocation pore component ISP42. A high copy number suppressor of temperature-sensitive isp42 has been isolated and sequenced. This novel gene, denoted *ISP6*, encodes a 61 amino acid integral membrane protein of the mitochondrial outer membrane, which is oriented with its amino-terminus facing the cytosol. Disruption of the *ISP6* gene is without apparent effect in wild type yeast cells, but is lethal in temperature-sensitive isp42 mutants. Immunoprecipitation of the gene product, ISP42p, from mitochondria solubilized under mild conditions reveals a multi-protein complex containing ISP6p and ISP42p.

Key words: ISP6/ISP42/mitochondria/mitochondrial outer membrane protein/protein translocation

Introduction

When a lipid bilayer intervenes between the site of protein synthesis and the site of protein function, specific machinery is required to surmount the membrane barrier. Protein translocation across membranes requires two separate steps. The first step is a sorting or recognition event in which proteins to be moved across a specific membrane must be discriminated from all other cellular proteins. The second step is the physical movement of protein through the bilayer, utilizing specific protein translocation machinery. It is towards a further understanding of such transport apparatus in the mitochondrial outer membrane that this paper is addressed.

In the biogenesis of mitochondria, >90% of mitochondrial proteins must be imported from the cytosol (a few are synthesized within the organelle). Most of the mitochondrial proteins whose import has been studied are synthesized as precursors, differing from the mature proteins by the presence of additional amino acids at the amino-terminus, termed the 'presequence'. Such presequences have been shown to contain the information required for recognition or discrimination of mitochondrial proteins from other cellular proteins. The information present in the presequence is interpreted by specific import receptors on the mitochondrial surface and precursors subsequently engage the actual translocation machinery in the outer membrane. Very little is known about this machinery; however, some important progress has been made. Recent work in both the yeast *Saccharomyces cerevisiae* and the filamentous fungus *Neurospora crassa* has identified homologous integral membrane proteins in the outer membrane of mitochondria with all the properties expected of a component of the translocation pore itself (Baker *et al.*, 1990; Kiebler *et al.*, 1990). This protein, identified through independent methods as ISP42 in yeast and MOM38 in *Neurospora*, can be cross-linked to membrane spanning translocation intermediates, and antibodies to it block protein import into mitochondria *in vitro* (Vestweber *et al.*, 1989; Scherer *et al.*, 1990; Sollner *et al.*, 1992). In addition, the yeast protein has been shown to be essential for viability under any conditions (Baker *et al.*, 1990). These properties suggested to us that ISP42 could be used as the starting point in a genetic search for additional components of the translocation machinery; we describe the beginnings of such a search below. The abbreviation 'ISP42', standing for import site protein of 42 kDa, has been used to refer to both the protein itself and the gene which encodes it. For clarity, we will here use *ISP42* to refer to the gene, and ISP42p to denote the gene product.

Results

The first step in our genetic search was to generate temperature-sensitive mutants in *ISP42*. We chose an *in vitro* mutagenesis approach based on the polymerase chain reaction (PCR) (Leung *et al.*, 1989). By amplifying the ISP42 gene under conditions of low fidelity, we generated a library of random mutations and subsequently selected several alleles that allowed cell growth at 23°C but not at 37°C. Figure 1A demonstrates the temperature-sensitive growth phenotype conferred by six such alleles as well as a wild type control. Independence of these alleles was confirmed by sequencing (data not shown). Each ISP42p mutant generated in this manner contained multiple amino acid replacements. These six alleles are recessive, allowing normal growth in a heterozygote. On centromere plasmids all six alleles allow growth of the *ISP42* chromosomal deletion strain at 23 or 30°C, but not at 37°C. Subsequent work in this study was done primarily with one allele which we have designated as isp42-3.

To search for proteins interacting with ISP42p, we looked for high copy number suppressors of isp42-3, i.e. normal cellular genes that, when over-expressed, allow the growth of temperature-sensitive isp42 mutants at non-permissive temperatures. We reasoned that the stability or function of an isp42-3-containing translocation complex at the non-permissive temperature might be increased by increasing the concentration of other members of the complex. High copy number suppressors were isolated by transforming isp42-3 cells with a yeast genomic library contained on a high copy number, 2μ-based plasmid (see Materials and methods). Transformants able to grow at 37°C were isolated, and the plasmids conferring the phenotype were recovered and analyzed. As expected, we readily recovered plasmids containing a wild type copy of the *ISP42* gene. However,

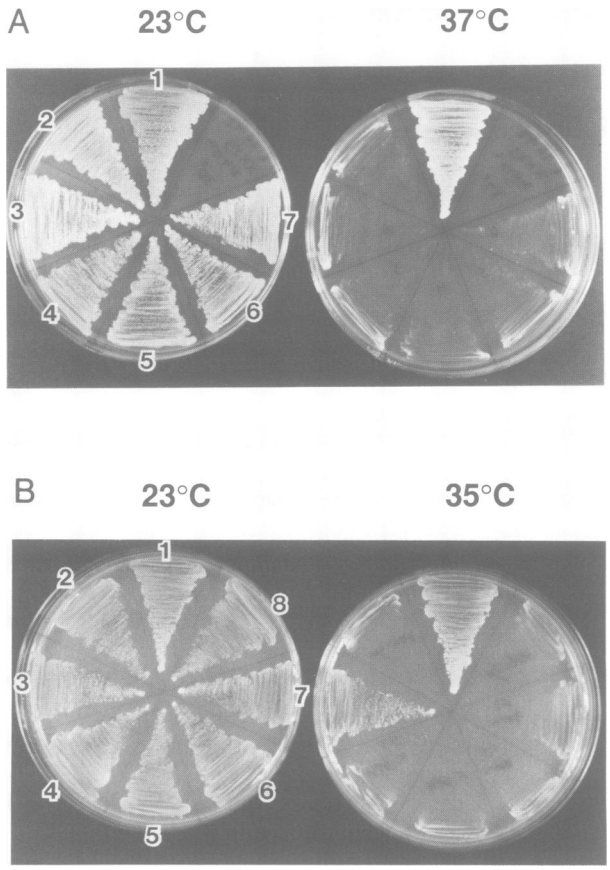

Fig. 1. Temperature-sensitive mutants of *ISP42* and high copy number suppression. (**A**) Shown are two Petri dishes inoculated identically with seven different yeast strains and subsequently incubated at the indicated temperatures. Sector 1 contains a strain (KKY3) bearing a wild type *ISP42* allele. Sectors 2–7 were inoculated with isogenic strains bearing six different mutant alleles of *ISP42* that allow growth at 23°C, but not at 37°C. (One sector contains no yeast.) (**B**) Shown are two Petri plates inoculated identically and subsequently incubated at the indicated temperatures. All sectors contain the temperature-sensitive strain KKY3-isp42-3 transformed with different plasmids from a yeast genomic library in the high copy number vector, YEp24. The yeast cells in sector 1 bear a plasmid containing the wild type *ISP42* gene. The yeast cells in sector 2 contain YEp24 lacking a genomic DNA insert. The yeast cells in sector 3 contain the plasmid 3S1, a high copy number suppressor of isp42-3. Sectors 4–8 contain yeast cells bearing various genomic clones that do not complement isp42-3.

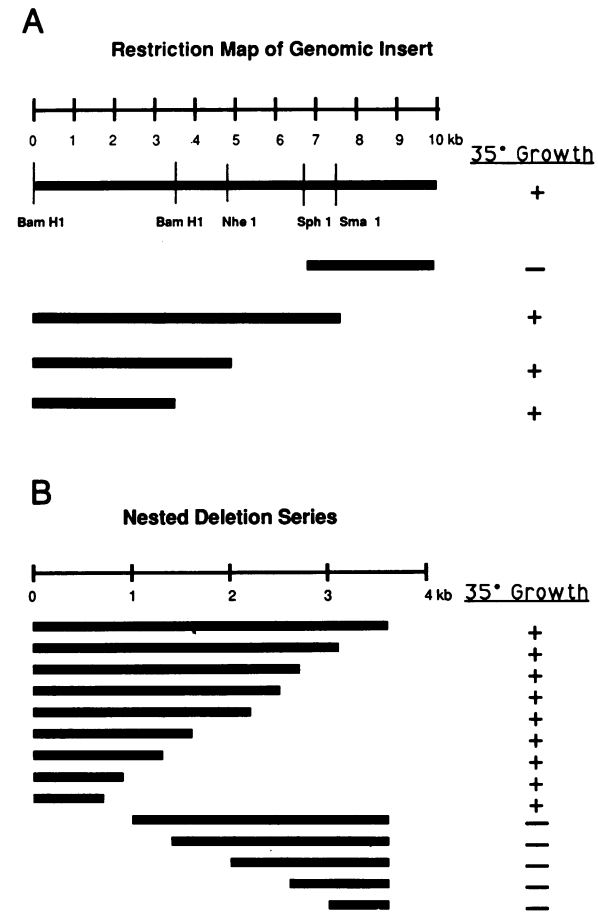

Fig. 2. Subcloning analysis of the high copy number suppressor 3S1. (**A**) Shown is an approximate restriction map of the genomic DNA insert of the plasmid 3S1. Beneath this map, dark bars indicate the DNA present in several smaller subclones. The growth characteristics at 35°C of KKY3-isp42-3 yeast bearing each subclone are shown to the right (+ indicates growth, – indicates lack of growth). (**B**) The 3.6 kb *Bam*HI fragment from 3S1 was subcloned into the vector p306-2μ, and nested deletions were generated using exonuclease III. The dark bars indicate the genomic DNA present in each of the resulting plasmids, and the growth characteristics at 35°C of KKY3-isp42-3 yeast bearing each plasmid are shown at the right (+ indicates growth, – indicates lack of growth).

by demanding growth at 37°C, we had difficulty recovering any plasmids that did not contain wild type *ISP42*. When we relaxed our growth requirements for the transformants to lower (but still non-permissive) temperatures, we were able to recover additional plasmids lacking the *ISP42* gene. In a screen of 10 000 transformants, 14 plasmids were recovered that allowed growth of isp42-3 yeast at 35°C. Of these, 11 contained wild type *ISP42* sequences and three did not. Two of the plasmids lacking *ISP42* were found to be identical by restriction mapping; we termed these plasmids 3S1 and their further analysis is described below. The third plasmid was unrelated and will be described elsewhere (C.K.Kassenbrock *et al.*, in preparation).

A 61 amino acid protein is a high copy number suppressor of isp42-3

The plasmid 3S1 was found to contain an insert of genomic DNA of ~10 kb. We subcloned various smaller fragments of this insert and then tested their ability to complement the 35°C growth defect of isp42-3 when present at a high copy number. A restriction map of the genomic insert and the growth characteristics of several subclones are shown in Figure 2A. We found that a 3.6 kb *Bam*HI fragment was necessary and sufficient to allow growth at 35°C. This *Bam*HI fragment was then subcloned into the vector p306-2μ, which allowed both the generation of nested deletions using exonuclease III digestion and the subsequent functional testing of these deletions as high copy number suppressors in yeast (see Materials and methods). As shown in Figure 2B, full complementing activity was obtained with genomic DNA of <700 bp derived from the left end of the original genomic insert as diagrammed here. We next sequenced this complementing DNA and looked for open reading frames (ORFs). The reading frame analysis is shown in Figure 3A. To our surprise, a 680 bp *Nsi*I–*Bam*HI fragment capable of high copy number suppression contained no large ORFs; however, two very small reading frames encoding 58 and 61 amino acids (aa) were present. To decide

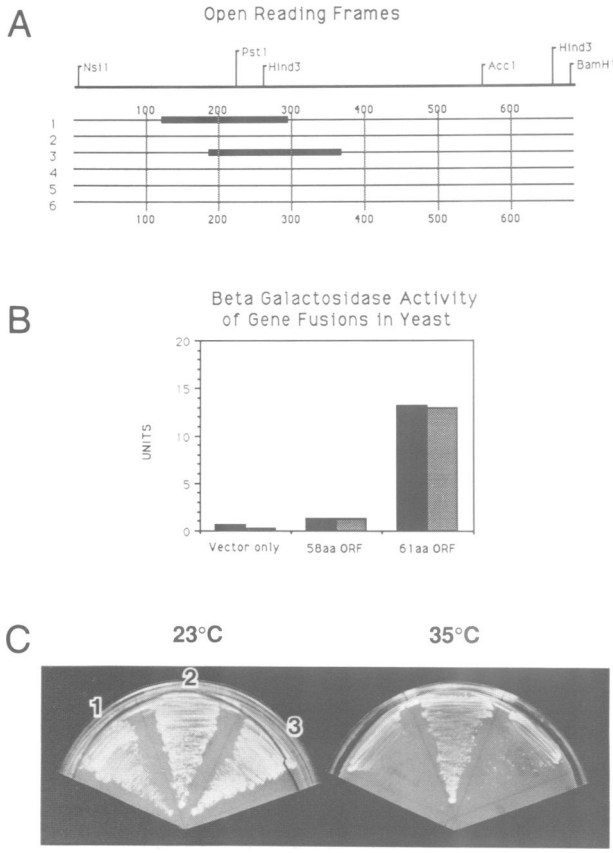

Fig. 3. A very small ORF is responsible for the high copy number suppression of isp42-3 by 3S1. (**A**) Shown is a restriction map of a 680 bp *NsiI*−*Bam*HI fragment from 3S1 that is sufficient for high copy number suppression of isp42-3. Underneath this map, dark bars indicate ORFs larger than 50 aa in each of the six possible reading frames. Frame 1 contains a 58 aa ORF and frame 3 contains a 61 aa ORF. (**B**) Gene fusions between the two ORFs shown above and the *E.coli lacZ* gene were made and introduced into yeast in the vector pSEY101 (Emr *et al.*, 1986). Each construct relies on the yeast sequences for promoter function and initiation of translation. Translation, if it occurs, would proceed through each complete reading frame (the stop codons have been deleted) and then into the *lacZ* gene (beginning with aa 9 of β-galactosidase). Two independent yeast transformants containing each construct as well as the vector alone (which lacks promoter and initiation methionine) were grown to mid log phase and permeabilized, and total β-galactosidase activity was measured. Shown is a graph of β-galactosidase activity; significant activity is associated only with the 61 aa ORF construct. (**C**) DNA encoding only the 61 aa ORF was obtained by PCR (see Materials and methods) and subcloned into the expression vector pMA91 (Mellor *et al.*, 1983). Yeast transformants of the temperature-sensitive strain KKY3-isp42-3 were inoculated onto two identical Petri dishes and incubated at the indicated temperatures. Sector 1 contains yeast transformed with pMA91 vector lacking insert. Sector 2 contains yeast transformed with pMA91 containing the 61 aa ORF, which is seen to complement isp42-3. Sector 3 contains yeast bearing pMA91 vector with the 61 aa ORF cloned in the reverse (non-coding) orientation.

if either of these small reading frames was expressed in yeast, we constructed gene fusions in which the entire reading frame of each was fused in-frame with the *Escherichia coli lacZ* gene. The resulting constructs have the potential to produce β-galactosidase fusion proteins but rely completely on the yeast sequences for promoter and initiation function. As a control the host vector pSEY101 (Emr *et al.*, 1986) alone was used which lacks both the promoter and the initiation methionine for the *lacZ* gene. These constructs were then introduced into yeast and the β-galactosidase activity within the resulting transformants was measured. As seen in Figure 3B, we found that the gene fusion containing the 61 aa ORF produced significant levels of β-galactosidase activity in yeast, indicating that this reading frame does encode a protein that is expressed in yeast. In contrast, the gene fusion containing the 58 aa ORF led to essentially background levels of β-galactosidase activity in yeast transformants. In *E.coli* both the 58 and the 61 aa gene fusion constructs (but not the vector alone) produced significant β-galactosidase activity, indicating that both fusion proteins retained enzymatic activity (not shown).

As a further test to confirm that the 61 aa ORF was fully responsible for the complementation of the 35°C growth defect of isp42-3 by the 3S1 plasmid, we constructed an expression plasmid in which DNA capable of encoding only the 61 aa ORF was cloned into an expression vector. We used PCR to add *Bam*HI sites immediately before the initiation methionine and after the stop codon and inserted the resulting 0.2 kb *Bam*HI fragment between PGK promoter and termination sequences using the vector pMA91 (Mellor *et al.*, 1983). As can be seen in Figure 3C, this 61 aa ORF expression construct does allow growth of isp42-3 at 35°C. Control transformants containing the expression vector alone or vector with the 61 aa ORF DNA in the reverse orientation are unable to grow at 35°C (Figure 3C).

Having thus identified the correct gene product, we examined it in more detail. Figure 4A shows the complete nucleic acid sequence of the gene as well as the predicted amino acid sequence. We term this gene *ISP6* to designate an import site protein of 6.4 kDa in recognition of its association with *ISP42*, which will be documented in more detail below. No significant homology was seen between *ISP6* and other sequences in the databases, nor were any recognizable motifs evident. However hydrophilicity analysis (Kyte and Doolittle, 1982), shown in Figure 4B, suggests that this 6.4 kDa protein is fairly hydrophobic in character and in particular contains a hydrophobic region (residues 32−52) with the potential to be a membrane-spanning domain.

The 61 amino acid protein, ISP6p, is essential for viability in isp42-3

To try to assess the function of this small 61 aa protein in yeast we constructed a gene disruption by inserting the *HIS3* gene into the *Pst*I site within the ORF (see Materials and methods for details). This construction was introduced into the genome of diploid yeast and His$^+$ haploids containing the disrupted *ISP6* gene were obtained by tetrad dissection. Correct integration was confirmed by PCR (data not shown). We were unable to detect any gross phenotype associated with the *ISP6* gene disruption in otherwise wild type yeast. In particular, there was no obvious effect on the growth rate of this strain on any carbon source tested (including the non-fermentable substrate, glycerol), or at any temperature tested (18, 23, 30 or 37°C).

Our identification of this small gene by high copy number suppression of isp42-3 mutants suggests that the two gene products interact. To look for additional genetic evidence for such an interaction, we attempted to construct a double

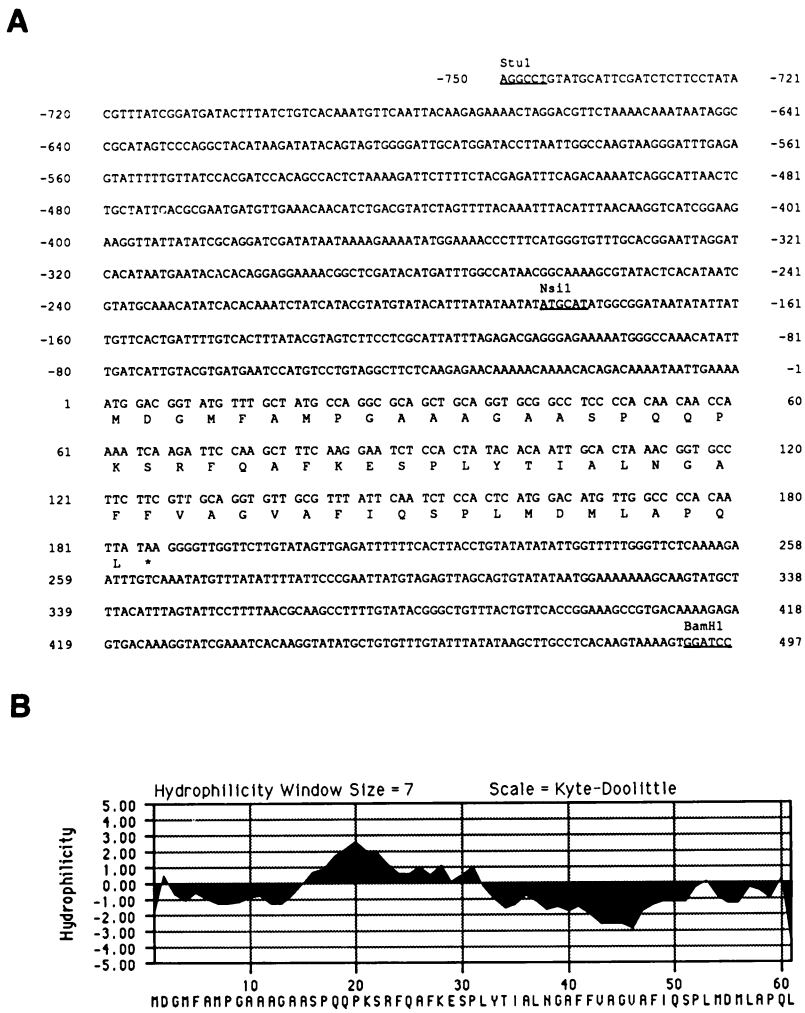

Fig. 4. Sequence analysis of the gene encoding the 61 aa protein, which we designate ISP6p. (**A**) Nucleotide sequence of a 1.25 kb *StuI*−*Bam*HI fragment containing the *ISP6* gene, a high copy number suppressor of isp42-3. A smaller 680 bp *NsiI*−*Bam*HI fragment is sufficient for complete gene function; this *NsiI* site at position −185 is underlined. Numbering begins at the translational start site and the predicted amino acid sequence is indicated in one letter code underneath the coding sequence. (**B**) Hydrophilicity analysis of the predicted amino acid sequence of ISP6p. Hydrophilicity was calculated by the method of Kyte and Doolittle (1982) and is shown graphically above the sequence. Positive values denote hydrophilic regions, negative values denote hydrophobic regions. The hydrophobic region between residues 32 and 52 is predicted to be a membrane-spanning domain. The complete nucleotide sequence of the 1.25 kb *StuI*−*Bam*HI fragment containing the *ISP6* gene has been submitted to GenBank under the accession number Z22815.

mutant of isp42-3 in combination with the disrupted *ISP6* gene. As shown in Figure 5 such a double mutant was inviable at any temperature but could be rescued by either the wild type *ISP42* or *ISP6* gene in single copy at 30°C. This finding of synthetic lethality is strong additional evidence for a physical interaction between both gene products (for discussion see Huffaker *et al.*, 1987).

ISP6p is an integral component of the mitochondrial outer membrane

To look for a physical interaction between the two proteins ISP42p and ISP6p by more direct methods, we generated antibodies to ISP6p. This was done by constructing a gene fusion between the *E.coli trpE* gene and the complete coding sequence of *ISP6*. Antibodies raised against the resulting fusion protein made in *E.coli* specifically recognized a low molecular weight protein present in mitochondrial fractions from wild type yeast cells (Figure 6A, lane 1, 'W.T.', arrow). This small protein is absent in mitochondria isolated from isogenic yeast containing the insertional gene disruption (Figure 6A, lane 2, 'isp6::HIS3'). The antiserum is also seen to react non-specifically with several bands of much higher apparent molecular weight present in equal amounts in the two strains (Figure 6A, both lanes). Other mitochondrial proteins such as ISP42p were also present at equal levels in mitochondria prepared from the two strains (not shown). The specific low molecular weight immunoreactive band migrates somewhat faster than the predicted size of 6.4 kDa; however, it comigrates exactly with ISP6p synthesized by *in vitro* translation (not shown).

The hydrophilicity analysis shown in Figure 4B predicts that ISP6p is hydrophobic and contains a membrane-spanning domain. To test this prediction, we extracted mitochondria with carbonate buffer at high pH to remove all soluble proteins and extrinsic membrane proteins. As shown in Figure 6B, ISP6p remains associated with the membrane under such conditions (compare lanes 2 and 3). In the presence of detergents, however, the protein is readily solubilized, confirming that it is an integral membrane protein (Figure 6B, lanes 5 and 6). A control integral

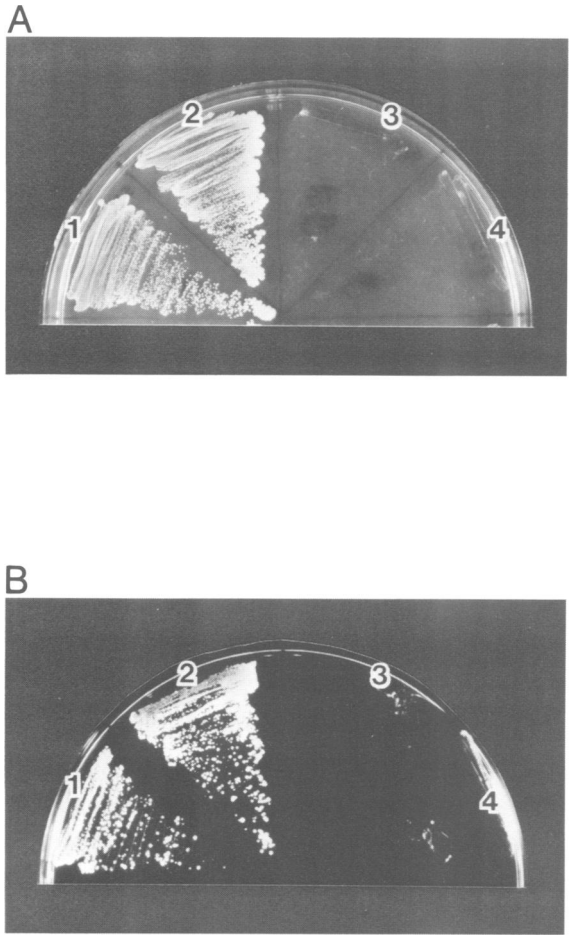

Fig. 5. Disruption of *ISP6* is lethal in an isp42-3 background. (**A**) A strain of yeast (KKY31-2) was constructed containing an insertional disruption of *ISP6* (*HIS3* inserted at the *Pst*I site within the coding sequence) and a complete coding sequence deletion of the chromosomal *ISP42* gene. The essential *ISP42* gene in this strain is provided by pRS316-ISP42, a centromere plasmid containing wild type *ISP42* and the *URA3* gene as selectable marker. This strain was transformed with a second centromere plasmid bearing TRP1 as the selectable marker and carrying either an additional wild type copy number of *ISP42* (pRS314-ISP42) or a temperature-sensitive allele of *ISP42* (pRS314-isp42-3). Transformants were then replated on selective medium containing 5-fluoroorotic acid (FOA) (Boeke *et al.*, 1987) which forces the loss of the *URA3*-containing pRS316-ISP42 and thus uncovers the allele present on the TRP1 plasmid. Shown is an FOA plate inoculated with two independent transformants for each TRP1 plasmid and incubated at the permissive temperature for isp42-3. Sectors 1 and 2 contain yeast bearing pRS314-ISP42, and sectors 3 and 4 contain yeast bearing pRS314-isp42-3, which are seen to be unable to grow even at the permissive temperature. (**B**) *ISP6* in single copy restores the viability of the *isp6*::*HIS3*, *isp42-3* double mutant. Transformants of the strain KKY31-2 described above containing pRS316-ISP42 and pRS314-isp42-3 were transformed with a third centromere plasmid containing *LEU2* as a selectable marker and either *ISP6* (pRS315-ISP6 which contains the 680 bp *Nsi*I–*Bam*HI fragment) or no insert (pRS315). Transformants were replated on selective FOA medium and incubated at the permissive temperature for isp42-3. Shown is an FOA plate inoculated with two independent transformants of each *LEU2* plasmid. Sectors 1 and 2 contain pRS315-ISP6, and sectors 3 and 4 contain pRS315. *ISP6* but not vector alone restores the ability of the double mutant (*isp6*::*HIS3*, *isp42-3*) to grow at the permissive temperature.

membrane protein of the mitochondrial inner membrane, the ADP/ATP carrier protein, exhibited identical extraction properties under these conditions (not shown).

We next sought to determine in which mitochondrial membrane ISP6p is located. The genetic analysis predicted that this protein should be associated with ISP42p. Previous analysis of ISP42p in *S.cerevisiae* and its homologue MOM38 in *N.crassa*, had determined that ISP42p is localized exclusively in the outer mitochondrial membrane and is intimately associated with mitochondrial precursors during transit into the organelle (Vestweber *et al.*, 1989; Kiebler *et al.*, 1990). In cell fractionation studies (not shown), we determined that all of the ISP6p present in the cell cofractionated with the ADP/ATP carrier (AAC2p) protein in mitochondria (not shown). In this fractionation analysis, we were able to show that the ratio of ISP6p to AAC2p in whole cell homogenates of yeast was the same as that in isolated mitochondria.

In order to define the membrane distribution of ISP6p in isolated mitochondria, the inner and outer membranes of mitochondria were separated on sucrose gradients and fractions were analyzed for the presence of ISP42p and the ADP-ATP carrier protein, in relation to ISP6p. As shown in Figure 6C, the inner and outer membranes are resolved by this procedure. The inner membrane marker, AAC2p, fractionates as a single dense peak. In contrast, the outer membrane marker, ISP42p, is resolved into two peaks, a dense peak overlapping with the inner membrane fraction, and a lighter peak free of inner membrane markers. The dense peak containing ISP42p presumably represents outer membrane fragments that are still tightly attached to the dense inner membrane at membrane contact sites, as has been described previously (Riezman *et al.*, 1983). Outer membrane fragments that are not tightly attached to the inner membrane are well resolved into a lower density peak. As seen in Figure 6C, ISP6p co-fractionates with ISP42p, indicating that it is an outer membrane protein.

The hydrophilicity analysis of Figure 4B suggests that residues 32–52 of ISP6p span the membrane, with the amino-terminal 31 residues on one side of the membrane and the carboxy-terminal nine residues on the other side. We sought evidence for the topology of the protein within the outer membrane by performing proteolysis studies. We reasoned that if the carboxy-terminus was oriented towards the cytosol, proteolytic treatment of intact mitochondria might result in a mobility shift of the protein, corresponding to the loss of nine amino acids. Alternatively, if the amino-terminus was exposed to the cytosol, more than half of the protein should be accessible to externally added protease, leading to a much larger mobility shift or even complete loss of our ability to detect the protein. We found that treatment of intact mitochondria with even low amounts of proteinase K led to the complete loss of detectable ISP6p by Western blot, suggesting that the amino-terminus is directed toward the cytosol (data not shown). It could be argued that our polyclonal anti-ISP6p antiserum might by chance only recognize an epitope at the carboxy-terminus of the protein. We feel that this is unlikely; however, if it were true, the observed data would indicate an orientation of ISP6p in the membrane opposite to that which we propose.

To determine the orientation unequivocally, we constructed epitope-tagged versions of ISP6p in which nine amino acids comprising the epitope recognized by the monoclonal antibody 12CA5 (Field *et al.*, 1988) were added to either the amino-terminus or the carboxy-terminus of ISP6p. Both tagged constructs were high copy number

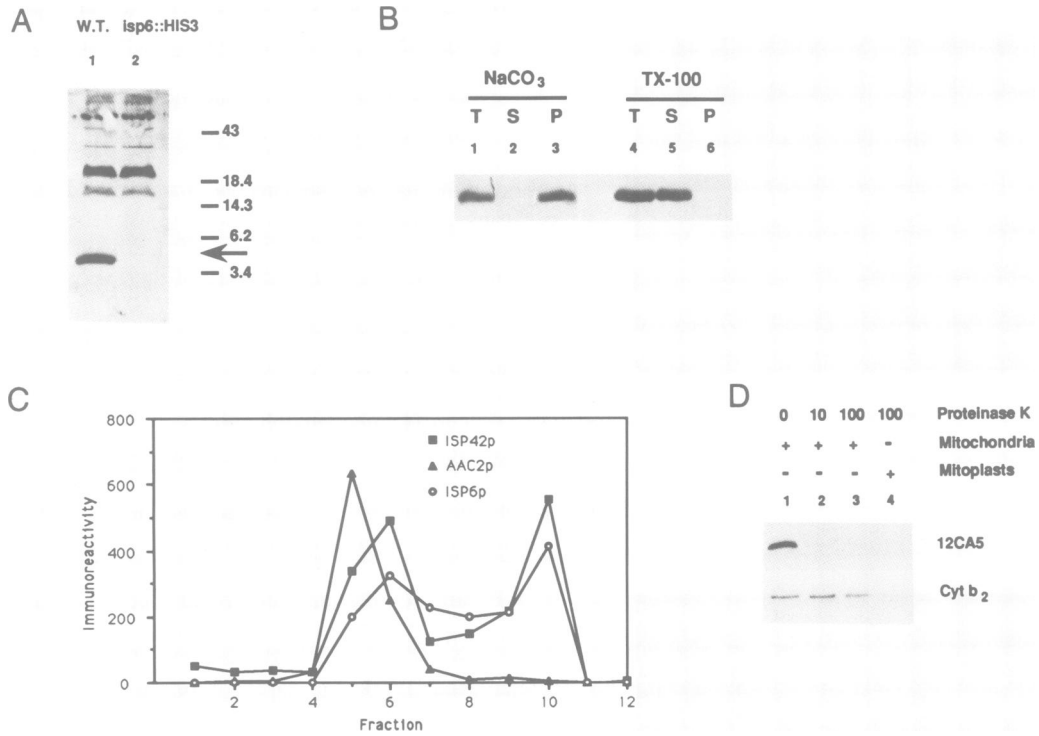

Fig. 6. ISP6p is an integral membrane protein of the mitochondrial outer membrane, oriented with its amino-terminus toward the cytosol. **(A)** ISP6p is a small protein present in mitochondria from wild type cells but missing in *isp6::HIS3* disruptants. Mitochondria were prepared from the wild type strain W303 and the isogenic strain KKY31 containing the *isp6:HIS3* gene disruption, subjected to electrophoresis on 16% polyacrylamide–SDS–tricine gels (Schagger and von Jagow, 1987), and then transferred to nitrocellulose for Western blotting. Antiserum against a TrpE–ISP6 fusion protein recognizes a specific low molecular weight protein (denoted by an arrow) present in wild type mitochondria (lane 1) but absent in mitochondria from the *isp6::HIS3* disruptant (lane 2). This serum also reacts non-specifically with several bands of higher molecular weight present at equal intensity in the two strains. **(B)** ISP6p is an integral membrane protein. Mitochondria prepared from wild type yeast were extracted with either 100 mM Na_2CO_3 or 1% Triton X-100 as indicated for 30 min on ice, centrifuged at 100 000 *g* for 1 h and separated into supernatant and pellet fractions. Fractions were then analyzed by Western blotting with ISP6p antibodies as in (A). 'T' indicates the total mitochondrial input, and 'S' and 'P' indicate the supernatant and pellet fractions after centrifugation. ISP6p is not extractable by carbonate but is completely solubilized by detergent. **(C)** ISP6p is localized to the mitochondrial outer membrane. Wild type mitochondria were subfractionated into inner and outer membrane fractions on 0.85–1.6 M sucrose gradients as described by Pon *et al.* (1989). Gradient fractions were analyzed with antisera to AAC2p and ISP42p by quantitative immuno dot blots (Jahn *et al.*, 1984) and with an antiserum against ISP6p by Western blotting. Immunoreactivity in arbitrary units is plotted against fraction number for each antibody. Fraction 1 is the bottom of the gradient and fraction 12 the top. ISP6p is seen to co-fractionate with the outer membrane marker, ISP42p. **(D)** The amino-terminus of ISP6p is exposed to the cytosol in intact mitochondria. Mitochondria were isolated from yeast expressing amino-terminal epitope-tagged ISP6 (see Materials and methods for details) and the mitochondria were treated with proteinase K for 30 min on ice. The protease was then inactivated by PMSF and the samples analyzed by Western blotting. In lane 1 are control mitochondria in which no protease was added. Mitochondria in lanes 2–4 were treated with either 10 μg/ml or 100 μg/ml of proteinase K, as indicated. In lane 4, the mitochondria were converted to mitoplasts by rupturing the outer membrane in hypotonic buffer before treatment with protease. Aliquots of each sample were analyzed by Western blotting with either the 12CA5 monoclonal antibody against the epitope tag or a polyclonal serum against the intermembrane space protein, cytochrome b_2, as indicated. The amino-terminal 12CA5 epitope on ISP6p is seen to be readily digested in intact mitochondria, whereas cytochrome b_2 is digested only after rupture of the outer membrane.

suppressors of isp42-3, indicating that the protein structures were not grossly perturbed by the epitope addition (not shown). Both tagged proteins showed the expected increase in size when cell extracts were analyzed by Western blotting with an anti-ISP6p antibody; however, for reasons not understood, only the amino-terminal construction was detectable using the 12CA5 antibody (not shown). We used this amino-tagged ISP6p construction in protease digestion experiments with isolated mitochondria to determine unambiguously the orientation of ISP6p within the outer membrane. Figure 6D shows that the 12CA5 epitope at the amino-terminus of ISP6p is accessible in intact mitochondria to protease added externally (compare lane 1 with lanes 2 and 3). In contrast, cytochrome b_2 in the intermembrane space of the same mitochondria is resistant to external protease, indicating that the outer mitochondrial membrane remains intact under the conditions of this experiment. Cytochrome b_2 can, however, be digested by protease when the outer membrane is deliberately ruptured by hypotonic swelling (lane 4). These data confirm the localization of ISP6p in the mitochondrial outer membrane and demonstrate that the amino-terminus is oriented towards the cytosol.

ISP6p exists in a stable complex with ISP42p

The genetic evidence presented earlier argues for a direct association between ISP6p and ISP42p. We have shown above that the intracellular location of the ISP6p is consistent with such an interaction. We next sought to demonstrate directly a physical interaction between the two proteins. It has been shown previously that solubilization of mitochondria with digitonin under very mild conditions allows the isolation by immune precipitation of a multiprotein outer membrane translocation complex containing ISP42p (or MOM38 in *Neurospora*) and several other identified proteins (Kiebler *et al.*, 1990; Moczko *et al.*, 1992; Sollner *et al.*, 1992). We used similar conditions to solubilize mitochondria from yeast

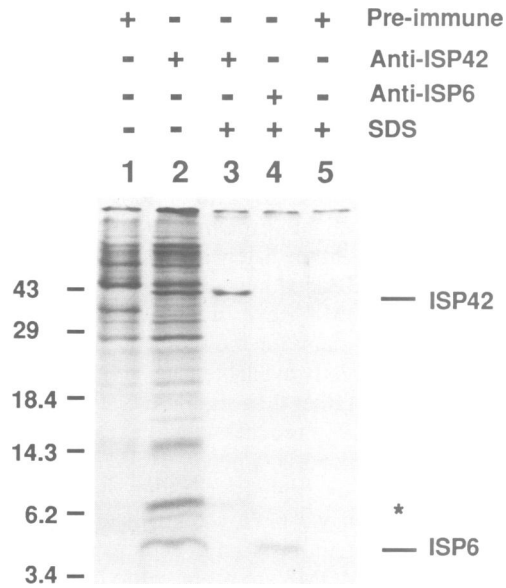

Fig. 7. ISP6p specifically co-precipitates with ISP42p. Yeast were metabolically labeled with ^{35}S and mitochondria were isolated and solubilized in 0.5% digitonin as described by Sollner *et al.* (1992) (see also Materials and methods). Solubilized mitochondria were then reacted with antibody to ISP42p (lane 2) or pre-immune serum (lane 1), precipitated with protein A–Sepharose and analyzed by SDS–PAGE. Shown is an autoradiogram of an SDS gel. Lane 1 reveals the non-specific background of proteins associated with protein A beads and pre-immune serum under these very mild washing conditions. Lane 2 shows the proteins precipitating when antiserum to ISP42p is used; ISP42p and several other specific proteins can be seen above the background. In lanes 3–5, immune complexes identical to those shown in lane 2 were further analyzed by disrupting the complexes in 1% SDS, 5% 2-mercaptoethanol-containing buffer at 100°C, and then diluting 10-fold with buffer containing 1% Triton X-100. The disrupted complexes were then reacted with various antibodies, reprecipitated with protein A–Sepharose and analyzed by SDS–PAGE. Lane 3 shows reprecipitation of the disrupted complex by ISP42p antiserum. As expected, ISP42p is again precipitated, this time essentially free of other proteins (interestingly, a small amount of a specific low molecular weight protein, marked by an asterisk, reproducibly reprecipitates with ISP42p). Re-precipitation of the disrupted complex with antibodies to ISP6p is shown in lane 4 and reveals that ISP6p is present in the complex. Reprecipitation of the disrupted complex by the pre-immune serum corresponding to anti-ISP6p reveals essentially no background bands under these more stringent washing conditions and confirms that the bands seen in lanes 3 and 4 are indeed specific.

which had been metabolically labeled with ^{35}S. Figure 7 shows immune precipitations from such mitochondria using antibodies directed against ISP42p or pre-immune serum. Under nondissociating conditions, antiserum against ISP42p co-immunoprecipitates from membrane extracts ISP42p as well as several other proteins. Notable among the low molecular weight proteins that coprecipitate with ISP42p is a band with a mobility similar to that of ISP6p. Another specific band, marked with an asterisk in the figure, is reproducibly observed in immunoprecipitations involving ISP42p antiserum. This may correspond to the yeast homolog of MOM8, a low molecular weight protein associated with the *Neurospora* complex (Sollner *et al.*, 1992). With the mild washing conditions employed in this experiment, some proteins associate non-specifically with the immunoadsorbent; these non-specific bands can be identified by their presence in the precipitate from pre-immune serum (lane 1).

To determine if the very low molecular weight protein which coprecipitated with ISP42p was in fact the 61 aa ISP6 protein, we took anti-ISP42p precipitates identical to those shown in lane 2, solubilized them under denaturing conditions with SDS, and reprecipitated them with either anti-ISP42p or anti-ISP6p antibodies. Reprecipitation using anti-ISP42p antibodies now results in immunoprecipitation of a single band of ISP42p, since the multi-protein complex was disrupted by SDS (Figure 7, lane 3). Immunoprecipitation from the detergent-disrupted complex using an antiserum against ISP6p specifically precipitates the low molecular weight band, identifying it as ISP6p (Figure 7, lane 3). Precipitation from the disrupted complex using pre-immune serum now reveals the absence of non-specific bands under these more stringent conditions (lane 5). In other studies, this protein complex containing both ISP42p and ISP6p can be isolated by antibodies directed against two different portions of the ISP42 protein, as well as by antibodies to ISP6p, confirming the specificity of the co-immunoprecipitation (data not shown). Densitometry of the ISP42p and ISP6p bands in lanes 3 and 4 of Figure 7 and in similar experiments yields a ratio of 1.1(±0.2):1.0, ISP6p:ISP42p after normalizing for the number of sulfur-containing residues in the two proteins, indicating a probable stoichiometry of 1:1 in the complex. This stoichiometry may be an underestimate of the complex which might exist between ISP6p and ISP42p in the membrane.

ISP6p is required for import by ISP42-3p at different temperatures

Deletion of *ISP6* failed to reveal any discernible phenotype on any carbon source tested. However, Δisp6 in combination with the isp42-3 temperature-sensitive mutant was inviable under these same conditions. In addition the overexpression of ISP6p was not able on its own to substitute for the complete loss of ISP42p. Based on these genetic studies and the cofractionation analysis, we reasoned that ISP6p probably interacts directly with ISP42p to stabilize its function or assembly in the membrane.

To examine the influence of ISP6p on the function of ISP42p, we measured *in vitro* protein import under conditions in which the activity of ISP42p was limiting. In control experiments (not shown), we observed that *in vitro* and *in vivo* import of different precursors was the same in wild type (W303) mitochondria and mitochondria lacking ISP6p. When import of a precursor to the F_1ATPase α subunit was examined in mitochondria prepared from the isp42-3 mutant (Figure 8), we observed that its rate of import is decreased by 85–90% at 25 and 37°C. However, if mitochondria are prepared from the same strain containing a 2μ *ISP6* plasmid (pISP6), we observe that import is restored to the level noted for wild type (W303) mitochondria. This decrease in isp42-3 and restoration in isp42-3 (pISP6) mitochondria occurs under conditions in which the level of ISP42 protein remains the same in the respective mitochondria preparations.

Discussion

In this study we have utilized a genetic search to identify components of the mitochondrial protein import machinery. Our approach has been to look for genetic interactions with

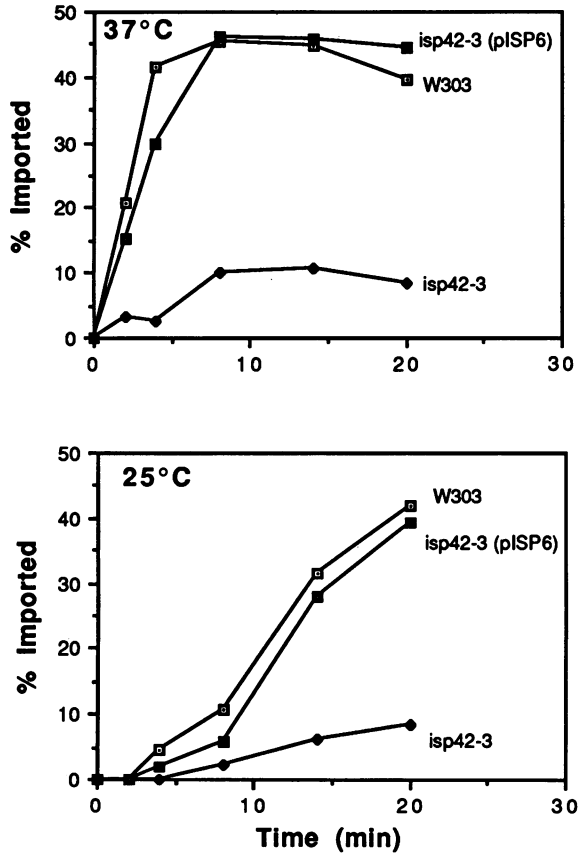

Fig. 8. Expression of *ISP6* in KKY3-isp42-3 restores the import competence of the mutant mitochondria *in vitro*. Mitochondria were prepared from three strains, KKY3-isp42-3 (isp42-3), KKY3-isp42-3+p306-2μ-ISP6 (isp42-3-pISP6) and W303 (wild type) from 30°C overnight cultures. During import, mitochondria were diluted to 300 μg per ml in import reaction buffer. Prior to adding reticulocyte lysate containing [^{35}S]methionine-labeled F1 α-subunit precursor, mitochondria were preincubated at import temperature for 20 min (upper panel, 37°C; lower panel, 25°C). After initiating import, 100 μl aliquots were removed at each time point and immediately transferred to a new tube containing 1 μl of 10 mg/ml valinomycin. After the time course, mitochondria were reisolated by centrifugation, lysed in SDS-containing sample buffer and resolved by SDS–PAGE. The level of the F1 α-subunit precursor and mature forms was quantified relative to an input standard by laser densitometry of fluorographs of the resulting gels.

the well characterized mitochondrial outer membrane translocation pore component, ISP42p (MOM38). We report here the construction of temperature-sensitive mutants in *ISP42* and the identification of *ISP6*, a high copy number suppressor of isp42-3. We show that null mutants in *ISP6* exhibit synthetic lethality in combination with isp42-3 and that *ISP6* encodes a 61 aa integral membrane protein of the mitochondrial outer membrane. We further demonstrate that ISP6p can be isolated in a stable complex with ISP42p by immune precipitation with ISP42p antibodies. These data establish a strong interaction between ISP42p and ISP6p and suggest therefore that ISP6p is a component of the translocation pore complex in the outer membrane.

Earlier studies have demonstrated a set of proteins in *Neurospora* that co-precipitate with antibodies against MOM19, a putative receptor for mitochondrial precursors in the mitochondrial outer membrane (Kiebler *et al.*, 1990; Sollner *et al.*, 1992). Notable in this complex are the presence of two small membrane proteins of 7 and 8 kDa (termed MOM7 and MOM8). The *Neurospora* complex also contains the ISP42p homolog (MOM38), MOM72 (another putative mitochondrial precursor receptor) and other proteins of 30 and 22 kDa apparent molecular weight. A qualitatively similar complex containing ISP42p has recently been described from yeast by Moczko *et al.* (1992). The presence of low molecular weight proteins in these putative translocation complexes immediately invites comparison with the 6.4 kDa protein described here, ISP6p. Since the genes for MOM7 and MOM8 have not yet been isolated, comparison with *ISP6* must for the moment rely solely on biochemistry. The currently available data from *Neurospora* emphasize differences rather than similarities. For example, we have shown that ISP6p is accessible to externally added protease in intact mitochondria, whereas MOM7 and MOM8 have been described as protease-resistant under similar conditions. In addition we have shown that ISP6p is an integral membrane protein, resistant to carbonate extraction, whereas MOM7 and MOM8 are proposed to be peripheral membrane proteins (Sollner *et al.*, 1992). It should be pointed out, however, that this proposal was based on the carbonate extractability of cross-linked products between the ADP/ATP carrier and MOM7 or MOM8 rather than the native proteins themselves and thus this data must be interpreted cautiously. The only other data available about the *Neurospora* proteins MOM7 and MOM8 is that they can be cross-linked to membrane-spanning translocation intermediates, which has led to the proposal that they line the import channel (Sollner *et al.*, 1992). We have no data yet regarding whether or not ISP6p can be cross-linked to similar membrane-spanning translocation intermediates.

The recently described yeast translocation complex has been analyzed in much less detail; the only additional finding of note here is that the yeast MOM7 was identifiable only by protein staining and not by ^{35}S-labeling, leading the authors to suggest that it contains no methionine or cysteine residues (Moczko *et al.*, 1992). In contrast, ISP6p contains five methionine residues and is readily ^{35}S-labeled (see Figure 7). This difference suggests that ISP6p is not MOM7. Could it be MOM8? We think not. MOM8 was named according to its apparent mobility on SDS–PAGE. We have shown here that ISP6p migrates faster than expected for its size rather than more slowly, as would be expected if it was identical to MOM8. Furthermore, our ISP42p immunoprecipitations reveal a more likely candidate for MOM8. Lane 2 of Figure 7 reveals a very prominent band (marked by an asterisk) which specifically co-precipitates with ISP42p and migrates above the 6.2 kDa marker, a mobility much closer to 8 kDa than the mobility of ISP6p. Interestingly, the association of this possible MOM8 homolog with ISP42p is particularly robust, in that we reproducibly find small amounts of it still co-precipitating with ISP42p after our attempts to disrupt the complex (Figure 7, lane 3, asterisk). Another specific band of slightly higher mobility (but still less than ISP6p), is of much less intensity on this ^{35}S-labeled gel and may perhaps be a candidate for MOM7. Taken together, the available data strongly suggest that ISP6p is not the yeast homolog of MOM7 or MOM8, but instead is a newly identified component of the protein translocation complex in the mitochondrial outer membrane.

Table I. Yeast strains used in this study

Strain	Genotype	Source
SEY6210	*MATα his3-Δ200 leu2-3,112 lys2-801 suc2-Δ9 trp1-Δ901 ura 3-52*	S.Emr[a]
SEY6211	*MAT***a** *ade2-101 his3-Δ200* leu2-3,112 suc2-*Δ9 trp1-Δ901 ura3-52*	S.Emr
W303	*MAT* **a**/*α ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1*	R.Rothstein[b]
D273-10B	*MATα*	(ATCC 24657)
KKY1	*MATα*/**a** *his3-Δ200/his3-Δ200 isp42::HIS3/ISP42 leu2-3,112/leu2-3,112 lys2-801/LYS2 ade2-101/ADE2 suc2-Δ9/suc2-Δ9 trp1-Δ901/trp1-Δ901 ura3-52/ura3-52*	K.Kassenbrock[c]
KKY2	*MATα his3-Δ200 isp42:HIS3 leu2-3,112 lys2-801 suc2-Δ9 trp1-Δ901 ura3-52 (pRS316-ISP42)*	K.Kassenbrock
KKY3	*MAT***a** *his3-Δ200 isp42::HIS3 leu2-3,112 ade2-101 suc2-Δ9* trp1-*Δ901 ura3-52* (pRS316-ISP42)	K.Kassenbrock
KKY31	*MAT***a** *ade2-1 can1-100 his3-11, 15 leu2-3,112 trp1-1 ura3-1 isp6::HIS3*	
KKY32	*MATα ade2-1 can1-100 his3-11, 15leu2-3,112 trp1-1 ura3-1 isp6::HIS3*	K.Kassenbrock
KKY31-2	*MATα ade2-1 his3 leu2-3,112 trp1 ura3 isp6::HIS3 isp42::HIS3 isp42::HIS3* (pRS316-ISP42)	K.Kassenbrock

[a]Emr *et al.* (1986).
[b]Wallis *et al.* (1989).
[c]This study.

The growing number of very small membrane proteins associated with the translocation complex invites speculation as to their function. We imagine that such small size precludes any extensive enzymatic activities and further suggests a structural role, perhaps in lining the translocation channel itself, as Sollner *et al.* (1992) have proposed. Alternatively, rather than forming part of the channel directly, the small channel-associated proteins might act to stabilize other channel-forming constituents. Mitochondrial import studies involving isp42-3 are consistent with this possibility. For example, the data presented here show that ISP6p stabilizes isp42-3p in some manner to reduce its thermal lability. A third possibility is that ISP6p and perhaps the other small proteins might be involved in some aspect of channel regulation or channel dynamics, for example allowing channel opening laterally for diffusion of membrane proteins out of the channel within the plane of the membrane, or helping to close or modulate the channel to prevent leakage during protein translocation or when not in use. One example is the proton-gating role for the epsilon subunit (61 aa peptide) of the proton-translocating ATPase of the mitochondrial inner membrane (Guelin *et al.*, 1993).

If ISP6p functions in a complex with ISP42p, why is disruption of the *ISP6* gene so well tolerated when disruption of *ISP42* is lethal? We do not know the answer, but there are two possibilities. The first is that the function performed by ISP6p is simply not required for growth of yeast under the conditions we have employed. The second possibility, which we consider more likely, is that ISP6p does perform an important function, but that other genes can also perform that function. Functional redundancy of *ISP6* could take the form of multiple genes encoding very similar proteins (as for example is the case with hsp70 proteins), or of different genes whose products perform similar roles (for example, perhaps MOM7 or MOM8 can suffice in ISP6p's absence).

The function of ISP6p in protein translocation is distinct from that of ISP42p. Deletion of the protein failed to reveal any detectable change in the import of different mitochondrial precursors both *in vitro* and *in vivo*. ISP6p on the other hand, even at a high copy number, was unable to substitute for loss of ISP42p. Only under conditions in which import required mutant ISP42p was the effect of *ISP6* in high copy number apparent. Since *ISP6*-dependent restoration of import occurred under conditions in which the levels of ISP42p remained unchanged, we believe that the small protein functions to stabilize ISP42p for its function in translocation. Further work will be required to elucidate the precise role of the newly identified ISP6p, and the roles of other small translocation complex-associated proteins.

Materials and methods

General

The yeast strains used in this study are shown in Table I. Yeast were grown on standard laboratory media as described by Sherman (1991). Yeast transformations were performed by the lithium acetate method of Schiestl and Gietz (1989). The procedure of Hoffman and Winston (1987) was used to isolate yeast DNA either for plasmid recovery in *E.coli* or to use as template DNA for PCR. Manipulations of DNA, including restriction digests, ligations and generation of nested deletions by exonuclease III digestion were performed by standard methods (Sambrook *et al.*, 1989). Sequencing was performed by the dideoxy method using Sequenase as directed by the manufacturer (US Biochemical). SDS–PAGE was performed using 16% gels and tricine-containing buffer according to the method of Schagger and von Jagow (1987). When large amounts of mitochondria or mitochondrial membranes (even carbonate-washed membranes) were run on our gels, a substance of high electrophoretic mobility which did not stain with Coomassie blue appeared to overload the gel near the dye front and distort the mobility of adjacent low molecular weight protein bands. This gel artifact could be avoided by precipitating samples with TCA prior to loading them on gels (acetone extraction was ineffective).

Construction of ISP42 genomic disruption strains

The *ISP42* gene was cloned by PCR from yeast genomic DNA using primers based on the published sequence (Baker *et al.*, 1990) that add *Sal*I sites at positions −334 and 1345 (5′-GCGGTCGACCTGACTGCCAGGGAC-ATGGGT and 5′-GCCGTCGACGAATTCCCCCTCAACTTGGTG). The 1.7 kb fragment obtained after PCR (1 min at 95°C followed by 30 cycles of: 30 s at 95°C, 30 s at 55°C and 90 s at 72°C; followed by a final 5 min at 72°C), was gel purified, cut with *Sal*I and cloned into pSP72 (Promega) to generate the plasmid pSP72-ISP42. pSP72-ISP42 was then cut with *Stu*I and *Sty*I to remove completely the *ISP42* coding region and a 1.77 kb *Bam*HI fragment containing the *HIS3* gene was ligated in its place after blunt ending the fragments with Klenow. The resulting plasmid, pISP-HIS, was cut with *Sal*I and the 2.2 kb fragment was gel purified and used to transform the diploid yeast strain SEY6210/6211. His$^+$ colonies were screened by PCR and Southern blotting to confirm correct integration of *HIS3* into the genomic *ISP42* locus; the resulting diploid strain was termed KKY1. Haploid strains KKY2 and KKY3 were generated from KKY1 as follows. pRS316-ISP42 was constructed by subcloning the 1.7 kb *Sal*I fragment containing the *ISP42* gene (described above) into the centromere plasmid pRS316 (Sikorski and Hieter, 1989), which contains the selectable marker *URA3*. This plasmid was transformed into KKY1 and the resulting Ura$^+$ transformant was sporulated to generate KKY2 and KKY3, haploid His$^+$ strains containing a complete coding sequence deletion of the genomic *ISP42* gene and a wild type *ISP42* gene on a centromere plasmid.

Generation of temperature-sensitive ISP42 mutants

Temperature-sensitive alleles of *ISP42* were generated by a modification of the low fidelity PCR technique of Leung *et al.* (1989). PCR was performed with the primers 5′-GGACCTCTCGAGCTGACTGCCAGGGAC and 5′-CGCCACGGATCCCTCAACTTGGTGCCC, using pSP72-ISP42 as template DNA (1 μl of miniprep DNA, ~100 ng, per 100 μl reaction). These primers generate a 1.7 kb product containing the *ISP42* gene with an *Xho*I site added at position −335 and a *Bam*HI site added at position 1336. The buffer composition was 50 mM KCl, 10 mM Tris−HCl, pH 8.3 at 25°C, 1.0 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, and could be made as a 10 × stock. $MnCl_2$ was added to a final concentration of 0.5 mM just before PCR, to avoid oxidation of Mn^{2+} at alkaline pH. Four separate PCR reactions were performed in which the concentration of one dNTP was reduced 5-fold with respect to the others. For example, in the low dATP reaction, dATP was at a final concentration of 40 μM, whereas dCTG, dGTP and dTTP were all at 200 mM. PCR was performed for 30 cycles with 2.5 U of *Taq* polymerase and the temperature cycling parameters stated above. The products from the four reactions were pooled, and the 1.7 kb band was gel purified and further amplified by use as template DNA in a subsequent PCR reaction using standard conditions (1.5 mM $MgCl_2$, no $MnCl_2$, equimolar dNTPs at 200 μM). The product was again gel purified, cut with *Xho*I and *Bam*HI, and ligated into the TRP1-containing centromere plasmid, pRS314 (Sikorski and Hieter, 1989), cut with the same enzymes. The ligation mix was amplified by transforming *E.coli*, scraping the transformants off Petri plates, and isolating miniprep DNA. The resulting mutant DNA library was used to transform KKY3.

Trp^+ transformants were replica-plated onto FOA plates (Boeke *et al.*, 1987) and incubated at 23 and 37°C. Colonies growing on FOA at 23°C but not 37°C were selected for further analysis, which included complementation of the 37°C growth defect by the wild type allele on pRS316-ISP42 and rescue of the mutant plasmid in *E.coli* followed by retransformation into KKY3 with re-establishment of the phenotype. Six independent temperature-sensitive alleles were isolated and designated isp42-1 to isp42-6. Independence was confirmed by restriction analysis and sequencing.

Selection of high copy number suppressors

KKY3-ts#3 was transformed with a yeast genomic library consisting of *Sau*3A partially digested genomic DNA cloned into the *Bam*HI site of the 2μ vector YEp24 (Carlson and Botstein, 1982). Following transformation, plates were incubated at 23°C overnight, then shifted to 35°C until colonies were formed. Usually one plate was left at 23°C to monitor transformation efficiency. Colonies growing at 35°C were restreaked and grown again at 35°C, then plasmids were recovered and retransformed into KKY3-ts#3 to confirm phenotype. Plasmids allowing growth at the non-permissive temperature were screened for the presence of *ISP42* by restriction analysis and PCR. Plasmids lacking *ISP42* sequences were analyzed further.

Construction of p306-2μ

The 2.2 *Eco*RI fragment from YEp24 (Botstein *et al.*, 1979) which contains the 2μ origin of replication was filled in with Klenow and ligated into the *Aat*II site of pRS306 (Sikorski and Hieter, 1989), which had previously been blunted with Klenow.

Construction of lacZ gene fusions and β-galactosidase assays

Gene fusions between the 58 and 61 aa ORFs and the *E.coli lacZ* gene were made as follows. PCR was performed with the primers 3S1-12 (5′-TGAATACACACAGGAGGA) and either 58ORF (5′-GCGGGATCCAATTGTGTATAGTGGAG) or 61ORF (5′-GCGGGATCCAATTGTGGGGCCAACAT) for 20 cycles using an annealing temperature of 50°C. Template consisted of 100 ng of DNA of the plasmid pBS-3S1-Bam-Stu which was constructed as follows. The 1.25 kb *Stu*I−*Bam*HI fragment from 3S1 containing the complete *ISP6* gene was subcloned into pBluescript II KS− (Stratagene) cut with *Eco*RV and *Bam*HI. The PCR reaction products were gel purified, cut with *Nsi*I and blunt ended with Klenow, then cut with *Bam*HI. The resulting fragments contain yeast genomic DNA sequences beginning at the *Nsi*I site at nucleotide −180 and extending to nucleotide 109 for the 58ORF and nucleotide 182 for the 61ORF, followed by the addition of a *Bam*HI site in-frame with the site in the vector pSEY101 (Emr *et al.*, 1986). These fragments were ligated into pSEY101 which had been cut with *Eco*RI, filled in with Klenow, then cut with *Bam*HI. The resulting construct pSEY101-58 has the potential to encode a fusion protein consisting of the first 57 aa of the 58 aa ORF followed by Asp, Pro and then the entire *lacZ* gene minus its first eight amino acids. The construct pSEY101-61 contains the same upstream DNA but would encode a fusion protein consisting of the entire 61 aa ORF followed by Asp, Pro and then the *lacZ* gene minus its first eight amino acids. The vector pSEY101 itself contains no promoter or initiation methionine for the *lacZ* gene.

β-Galactosidase assays were performed as follows. W303 cells transformed with pSEY101, pSEY101-58 or pSEY101-61 were grown in minimal selective medium containing glucose, and equivalent amounts of log phase cells were harvested. Cells were permeabilized with chloroform and SDS and total β-galactosidase activity was measured using *o*-nitrophenyl β-galactoside as a substrate, as described by Sambrook *et al.* (1989).

Construction of pMA91-61orf

PCR was performed with the primers 61-N-Bam (5′-GCCGGATCCAAAATGGACGGTATGTTT) and 61-C-Bam (5′-CGTGGATCCTTATAATTGTGGGGCCAA) and 100 ng of pBS-3S1-Bam-Stu as template. PCR parameters were as described above with 20 cycles and an annealing temperature of 55°C. The resulting 0.2 kb product consists of the coding sequence for the 61 aa ORF (from −3 to 186) flanked by *Bam*HI sites. This DNA was gel purified, cut with *Bam*HI and ligated into vector pMA91 (Mellor *et al.*, 1983) that had been cut with *Bgl*II and phosphatased. Plasmids containing the insert in both orientations were recovered, as determined by *Pst*I and *Pst*I + *Hin*dIII digestions.

Generation of ISP6 disruption strains

The plasmid pBS-3S1-Bam-Stu, described above, was linearized with *Pst*I and then ligated to the 1.7 kb *Bam*HI fragment containing the *HIS3* gene, after both fragments had been blunted with Klenow. The resulting plasmid, pISP6-HIS3 was then used as template DNA for PCR using M13(−20) forward (5′-GTAAAACGACGGCCAGT) and M13(−24) reverse (5′-AACAGCTATGACCATG) sequencing primers (30 cycles, 55°C annealing temperature, other parameters as described above) to generate a 3 kb product. The PCR DNA was extracted with phenol, ethanol precipitated and used to transform the diploid yeast strain, W303. His^+ transformants were selected and screened for correct integration of the *HIS3* gene into the *ISP6* locus by PCR using the *ISP6*-specific primers 3S1-7 (5′-CCAACATGTCCATGAGTG) and 3S1-11 (5′-TACGTAGTCTTCCTCGCA) with genomic DNA as template. The resultant diploid strain was sporulated and His^+ spores selected to obtain the haploid strains, KKY31 and KKY32, containing the *ISP6* gene disruption. The strain KKY31-2 was made by mating KKY31 with KKY2 and screening spores for the presence of both *ISP42* and *ISP6* genomic disruptions by PCR.

Generation of antibodies

Antibodies to ISP42p and ISP6p were raised against fusion proteins produced in *E.coli*. Gene fusions that contained the yeast sequences fused to the *E.coli trpE* gene were made using the vector pATH3 (Koerner *et al.*, 1991). For ISP42p, three different fusion proteins were generated containing either the amino-terminal half, or the carboxy-terminal half, or the full length *ISP42* sequence fused to *trpE*. The amino-terminal construct pATH-ISP42-N was made by performing PCR with the primers ISP-N-5 (5′-GCGATCGGATCCATGTCTGCACCAACTCCA) and ISP-N-3 (5′-CCGGTCAAGCTTTCAGAATTCGCCCTTCTCAGA). The carboxy-terminal construct pATH-ISP42-C was made with a PCR product produced with the primers ISP-C-5 (5′-GCGATCGGATCCGAATTCACAGGTGTTGCT) and ISP-C-3 (5′-CCGGTCAAGCTTTCACAATTGAGGAAGAGC). The full length construct pATH-ISP42-F was made from PCR using primers ISP-N-5 and ISP-C-3. PCR was done for 30 cycles with an annealing temperature of 55°C and other parameters as described above. PCR products were gel purified, cut with *Bam*HI and *Hin*dIII and ligated into pATH3 that had been cut with the same enzymes. For *ISP6* the 0.2 kb PCR product produced with the primers 61-N-Bam and 61-C-Bam as described above (see pMA91-61orf construction) was ligated into pATH3 that had been cut with *Bam*HI and phosphatased, to generate pATH-3S1-61Bam. All pATH constructions were transformed into *E.coli* strain RR1 and induced as described by Koerner *et al.* (1991). Fusion proteins were excised from preparative SDS−polyacrylamide gels, electroeluted and used to immunize rabbits. All anti-ISP42p antibodies recognized a single 42 kDa band on Western blots of yeast extracts. Anti-ISP6p antibodies are characterized in Figure 6.

Isolation of mitochondria

Yeast were grown in semi-synthetic medium containing 2% lactate and mitochondria were isolated as described by Daum *et al.* (1982). Isolated mitochondria were resuspended to 5 mg/ml in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2), frozen in liquid nitrogen and stored at −70°C.

Carbonate and detergent extraction of mitochondria

60 μg aliquots of isolated mitochondria from the wild type strain D273-10B were pelleted and resuspended in 100 μl of either 0.1 M Na_2CO_3 (pH 11.0–11.5) or 1% Triton X-100, 0.5 M NaCl, 20 mM HEPES, 5 mM EDTA, 1 mM α_2-macroglobulin, 2 mM PMSF, 2 mM *o*-phenanthroline, and incubated for 30 min on ice. The samples were then sedimented at 100 000 *g* for 60 min to generate pellet and supernatant fractions. The pellets were resuspended in 0.5 ml of 20 mM HEPES, 1 mM EDTA, 1 mM PMSF, pH 7.4, and the supernatants were diluted to the same volume in the same buffer. 10 μg of BSA was added as carrier to all fractions and they were precipitated by the addition of TCA to 5% for 20 min on ice, followed by centrifugation for 10 min in a microfuge. Samples were then solubilized in SDS sample buffer, and analyzed by Western blots.

Mitochondrial subfractionation

Mitochondria were fractionated into inner and outer membranes essentially as described by Pon *et al.* (1989). Mitochondria were resuspended at 10 mg/ml in SEM buffer, then diluted with 9 vol of 20 mM HEPES, 0.5 mM EDTA, 1 mM PMSF, pH 7.4 and incubated for 30 min on ice. After hypotonic swelling, the mitochondria were shrunken by the addition of 0.33 vol of 1.8 M sucrose, 10 mM KCl, 5 mM HEPES, 8 mM $MgCl_2$, 8 mM ATP, pH 7.4. After 10 min on ice, the mitochondria were sonicated on ice with three pulses of 30 s at 80% output using a Kontes Micro Ultrasonic Cell Disrupter equipped with a microprobe. Large fragments and unbroken mitochondria were cleared by centrifugation at 29 000 g_{max} for 20 min at 4°C and submitochondrial vesicles pelleted from the supernatant by centrifugation at 200 000 g_{max} for 45 min at 4°C. The submitochondrial vesicles obtained from 30 mg of mitochondria were resuspended in 100 μl of SEM and layered on top of 4 ml linear 0.85–1.6 M sucrose gradients containing 10 mM KCl, 5 mM HEPES, 1 mM $MgCl_2$, pH 7.4. The gradients were spun for 16 h at 121 000 g_{max} (30 k.r.p.m. in an SW60 rotor) at 2°C. A prominent orange membrane band was visible about one-third of the way from the bottom of the tube, and a faint whitish-yellow band was visible near the top. Gradients were fractionated by puncturing the bottom of the tubes.

Construction of epitope-tagged ISP6

A gene construction was made in which DNA encoding a 9 amino acid epitope (YPYDVPDYA) of influenza virus hemagglutinin recognized by the monoclonal antibody 12CA5 (Field *et al.*, 1988) was inserted into *ISP6* just following the initiation methionine codon. This was done as follows: PCR was performed with the primers N-EPI-5′ (5′-CAAAATAATTGA-AAAATGTACCCATACGATGTTCCAGATTACGCTGACGGTATGTT-TGCTATG) and M13(−20) forward, using 100 ng of pBS-3S1-Bam-Stu as template, for 30 cycles with an annealing temperature of 45°C. The product of this reaction was gel purified and designated 'product 1'. A second PCR reaction was performed using the primers N-EPI-3′ (5′-CATAGC-AAACATACCGTCAGCGTAATCTGGAACATCGTATGGGTACATT-TTTCAATTATTTTG) and M13(−24) reverse, and 100 ng of pBS-3S1-Bam-Stu as template, as above. The product from this reaction was gel purified and designated 'product 2'. A third PCR reaction was then performed in which the template DNA consisted of ~50 ng each of product 1 plus product 2, and the primers were M13(−20) forward and M13(−24) reverse. PCR was done for 20 cycles with an annealing temperature of 55°C, then the product of this third PCR reaction was gel purified, cut with *Bam*HI and *Xho*I and ligated into p306-2μ cut with the same enzymes, to generate the plasmid p306-2μ-3S1-N-EPI, which was transformed into yeast.

Proteinase K digestions

Mitochondria were prepared as described from yeast expressing epitope-tagged ISP6p. For digestion of intact mitochondria, 10 μl of mitochondria (40 μg) in SEM buffer was first diluted 10-fold in SEM buffer on ice. For digestion of mitoplasts, the same amount of mitochondria was diluted 10-fold in hypotonic buffer (20 mM HEPES–KOH, 5 mM EDTA, pH 7.4). Proteinase K was added to either 10 or 100 μg/ml final concentration as indicated and the samples incubated for 30 min on ice. Proteinase K was inactivated by adding PMSF to 2 mM, followed by TCA precipitation. Samples were then solubilized in SDS sample buffer and analyzed by Western blotting.

^{35}S-labeling of yeast and immunoprecipitations

Wild type yeast (strain D273-10B) was grown overnight on low sulfate medium containing 2% lactate and 100 μCi/ml $Na_2{}^{35}SO_4$. Cells were harvested at an OD_{600nm} of 0.5–1.0 and mitochondria were isolated. ^{35}S-labeled mitochondria were solubilized in 0.5% digitonin, 250 mM sucrose, 200 mM NaCl, 20 mM HEPES, 10% glycerol, 1% BSA, 1 mM EDTA, 1 mM PMSF, pH 7.4 for 30 min on ice (similar to Sollner *et al.*, 1992). Solubilized mitochondria were incubated with different antisera on ice for 2 h followed by incubation with protein A–Sepharose CL-4B beads for 1 h. Protein A beads were washed by centrifugation through two 30% sucrose cushions in the same buffer. Samples were then boiled in SDS sample buffer and either loaded directly on gels or subjected to a second round of immunoprecipitation. For the second immunoprecipitations, samples in SDS were diluted 10-fold with buffer containing 1% Triton X-100, 20 mM HEPES, 5 mM EDTA, pH 7.4, before the second incubation with antisera and protein A beads.

In vitro protein import into isolated mitochondria

The *E.coli* strain MC1066 and plasmid pT_7ATP1 (Takeda *et al.*, 1986) containing the gene encoding for the precursor to F1-ATPase α-subunit were used. *In vitro* transcription and translation as well as *in vitro* import assays were done as described previously (Cyr and Douglas, 1991) except that 25 mM creatine phosphate and 2.5 mg/ml of creatine phosphokinase were replaced with 10 mM potassium phosphate (monobasic). Mitochondria (300 μg/ml) were preincubated at import temperature for 20 min prior to import. SDS–PAGE, fluorography and quantification of fluorographs were also done as described previously (Cyr and Douglas, 1991).

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References

Baker,K.P., Schaniel,A., Vestweber,D. and Schatz,G. (1990) *Nature*, **348**, 605–609.

Boeke,J.D., Trueheart,J., Natsoulis,G. and Fink,G.R. (1987) *Methods Enzymol.*, **154**, 164–175.

Botstein,D., Falco,S.C., Stewart,S., Brennan,M., Scherer,S., Stinchcomb,D.T., Struhl,K. and Davis,R. (1979) *Gene*, **8**, 17–24.

Carlson,M. and Botstein,D. (1982) *Cell*, **28**, 145–154.

Cyr,D.M. and Douglas,M.G. (1991) *J. Biol. Chem.*, **266**, 21700–21708.

Daum,G., Bohni,P.C. and Schatz,G. (1982) *J. Biol. Chem.*, **257**, 13028–13033.

Emr,S., Vassarotti,A., Garrett,J., Geller,B., Takeda,M. and Douglas,M. G. (1986) *J. Cell Biol.*, **102**, 523–533.

Field,J., Nikawa,J., Broek,D., MacDonald,B., Rodgers,L., Wilson,I.A., Lerner,R.A. and Wigler,M. (1988) *Mol. Cell. Biol.*, **8**, 2159–2165.

Guelin,E., Chevallier,J., Rigoulet,M., Guerin,B. and Velours,J. (1993) *J. Biol. Chem.*, **268**, 161–167.

Hoffman,C.S. and Winston,F. (1987) *Gene*, **57**, 267–272.

Huffaker,T.C., Hoyt,M.A. and Botstein,D. (1987) *Annu. Rev. Genet.*, **21**, 259–284.

Jahn,R., Schiebler,W. and Greengard,P. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 1684–1687.

Kiebler,M., Pfaller,R., Sollner,T., Griffiths,G., Horstmann,H., Pfanner,N. and Neupert,W. (1990) *Nature*, **348**, 610–616.

Koerner,T.J., Hill,J.E., Myers,A.M. and Tzagoloff,A. (1991) *Methods Enzymol.*, **194**, 477–490.

Kyte,J. and Doolittle,R.F. (1982) *J. Mol. Biol.*, **157**, 105–132.

Leung,D.W., Chen,E. and Goeddel,D.V. (1989) *Technique*, **1**, 11–15.

Mellor,J., Dobson,M.J., Roderts,N.A., Tuite,M.F., Emtage,J.S., White,S., Lowe,P.A., Patel,T., Kingsman,A.J. and Kingsman,S.M. (1983) *Gene*, **24**, 1–14.

Moczko,M., Dietmeier,K., Sollner,T., Segui,B., Steger,H.F., Neupert,W. and Pfanner,N. (1992) *FEBS Lett.*, **310**, 265–268.

Pon,L., Moll,T., Vestweber,D., Marshallsay,B. and Schatz,G. (1989) *J. Cell Biol.*, **109**, 2603–2616.

Riezman,H., Hay,R., Gasser,S., Daum,G., Schneider,G., Witte,C. and Schatz,G. (1983) *EMBO J.*, **2**, 1105–1111.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schagger,H. and von Jagow,G. (1987) *Anal. Biochem.*, **166**, 368–379.

Scherer,P.E., Krieg,U.C., Hwang,S.T., Vestweber,D. and Schatz,G. (1990) *EMBO J.*, **9**, 4315–4322.

Schiestl,R.H. and Gietz,R.P. (1989) *Curr. Genet.*, **16**, 339–346.

Sherman,F. (1991) *Methods Enzymol.*, **194**, 3–21.
Sikorski,R. and Hieter,P. (1989) *Genetics*, **122**, 19–27.
Sollner,T., Rassow,J., Wiedmann,M., Schlossmann,J., Keil,P., Neupert,W. and Pfanner,N. (1992) *Nature*, **355**, 84–87.
Takeda,M., Chen,W., Smagula,C. and Douglas,M.G. (1986) *J. Biol. Chem.*, **261**, 15126–15133.
Vestweber,D., Brunner,J., Baker,A. and Schatz,G. (1989) *Nature*, **341**, 205–209.
Wallis,J., Chrebet,G., Brodsky,G., Rolfe,M. and Rothstein,R. (1989) *Cell*, **58**, 409–419.

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